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Vasoregulation by the β1 subunit of the calcium-activated potassium channel

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Small arteries exhibit tone, a partially contracted state that is an important determinant of blood pressure. In arterial smooth muscle cells, intracellular calcium paradoxically controls both contraction and relaxation. The mechanisms by which calcium can differentially regulate diverse physiological responses within a single cell remain unresolved. Calcium-dependent relaxation is mediated by local calcium release from the sarcoplasmic reticulum. These 'calcium sparks' activate calcium-dependent potassium (BK) channels comprised of α and β1 subunits. Here we show that targeted deletion of the gene for the β1 subunit leads to a decrease in the calcium sensitivity of BK channels, a reduction in functional coupling of calcium sparks to BK channel activation, and increases in arterial tone and blood pressure. The β1 subunit of the BK channel, by tuning the channel's calcium sensitivity, is a key molecular component in translating calcium signals to the central physiological function of vasoregulation.

Chronic blood pressure elevation resulting from increased arterial tone creates a burden on many organs leading to stroke, heart disease and renal disease[1]. An understanding of the molecules involved in regulating arterial tone is crucial to improving our understanding of essential hypertension and perhaps to developing better therapies. Large-conductance calcium-activated potassium channels (BK channels) are pivotal in the regulation of arterial tone, where they facilitate a negative feedback mechanism which opposes vasoconstriction[2]. Intravascular pressure increases arterial tone by a complex process which includes a graded membrane depolarization and elevation of calcium influx through dihydropyridine-sensitive, voltage-dependent calcium channels[3–5]. Calcium influx causes a global increase in cytoplasmic calcium leading to vasoconstriction. Calcium influx also activates localized calcium release events from ryanodine receptors, termed calcium sparks, which in turn activate nearby calcium-activated potassium (BK) channels, causing a hyperpolarizing current to oppose vasoconstriction[6]. Arterial tone therefore results from the interplay of opposing calcium-dependent processes: constriction, which is driven by global increases in calcium; and relaxation, which is driven by localized calcium concentrations[6]. The important role of BK channels in smooth muscle is demonstrated when the channels are specifically blocked with iberiotoxin[7], which leads to marked membrane depolarization and vasoconstriction[6,8] and inhibits the actions of a variety of smooth muscle relaxants[6,9].

BK channels are broadly expressed, and have functional roles in vascular smooth muscle as well as other tissues including skeletal muscle, neurons, kidney and secretory cells[7,10–16]. The functional diversity required for the tissue-specific roles of BK channels may be created in part by association with accessory β-subunits. A family of four BK β-subunits has been identified[17–20]. Each family member has a different tissue distribution and different effects on BK channel pharmacology and activation gating. The β1-subunit is enriched in smooth muscle and purifies with the BK pore-forming subunit[21]. In expression systems, the β1 subunit confers an increased calcium sensitivity, slows gating kinetics and increases the sensitivity to the agonist dehydrosoyasaponin (DHS-1)[20,22–24]. Using sensitivity to DHS-1 as a probe for BK α/β1 subunits, it has been shown that human coronary artery smooth muscle is enriched for α/β1-assembled BK channels, and that these channels are more calcium sensitive than BK channels in other tissues where the β1 subunit is not expressed[25].

Although the functional role of the BK β1 subunit in native tissues is unknown, the assumption is that the β1 subunit may increase calcium sensitivity sufficiently for regulation of smooth muscle membrane properties. However, evidence indicates that BK channels are localized very close to calcium release sites, such that they would be exposed to effectively high calcium concentrations (>10 μM calcium)[26] during calcium release. Thus, it is not known whether the increased BK channel calcium sensitivity conferred by the β1 subunit contributes to normal smooth muscle function.

Our hypothesis was that the β1 subunit has a central molecular role in conferring specificity to the translation of local calcium signals to long-distance electrical events (that is, membrane hyperpolarization) so as to couple calcium to a decrease in vascular tone and blood pressure. We have used gene targeting to eliminate β1 subunit expression in mice and evaluate its contribution to vascular smooth muscle function. We examined the roles of the β1 subunit in determining the Ca^{2+} sensitivity of BK channels, and the coupling of Ca^{2+} sparks to BK channel activity in smooth muscle cells from cerebral arteries. Furthermore, the β1 knockout mice allowed us to evaluate the functional role of the β1 subunit in regulating arterial tone and systemic blood pressure in awake, resting animals.

Our results indicate that the calcium sensitivity of the BK channel is uniquely matched by β-subunit association to a given calcium signalling modality to modulate the physiology of arteries.

Generation of β1 knockout mice

To create a null allele of the β1 locus, the gene-targeting vector was constructed to delete the first coding exon (exon 2) of the gene[27]. Exon 2 encodes the amino-terminal coding sequence including the

first transmembrane domain of the β1 protein. The targeting vector was designed to insert a β-galactosidase reporter in translational frame with the β1 subunit translation initiation site (Fig. 1a), and thus report transcription from the β1 gene promoter. Transfection of the targeting construct in embryonic stem cells generated three independent clones that specifically targeted the mouse β1 gene. Each of the embryonic stem cell clones was implanted into blastocysts and produced germline transmitting mice. Genomic Southern analysis using DNA fragments to hybridize outside the left and right arms of the targeting vector confirmed the recombination (Fig. 1b), seen as a polymorphism that shifts the bands to a predictable, larger size (Fig. 1). To confirm the disruption of the β1 gene, an antisense RNA probe encompassing the N-terminal coding region was used as a probe against RNA extracted from β1 knockout and control stomach tissue. RNAse protection (Fig. 1c) reveals a protected β1 probe hybridizing to control but not knockout mice RNA, confirming the lack of β1 expression in the knockout mice.

β1 messenger RNA is enriched in smooth muscle

The β1 subunit mRNA has been detected in many tissues containing smooth muscle[27]. The lacZ gene was targeted to the β1 locus to permit the examination of the cell types that normally express the β1 subunit. Figure 2 shows lacZ staining in isolated cerebral arteries used in this study as well as other smooth muscle tissues. Within certain tissues, expression is restricted to arterial smooth muscle. Examples are brain and heart (Fig. 2c, g), where β1 expression is largely undetected except in the vasculature, such as the cerebral arteries (Fig. 2b), aorta and coronary arteries (not shown). This is consistent with *in situ* hybridizations of β1 RNA, where expression

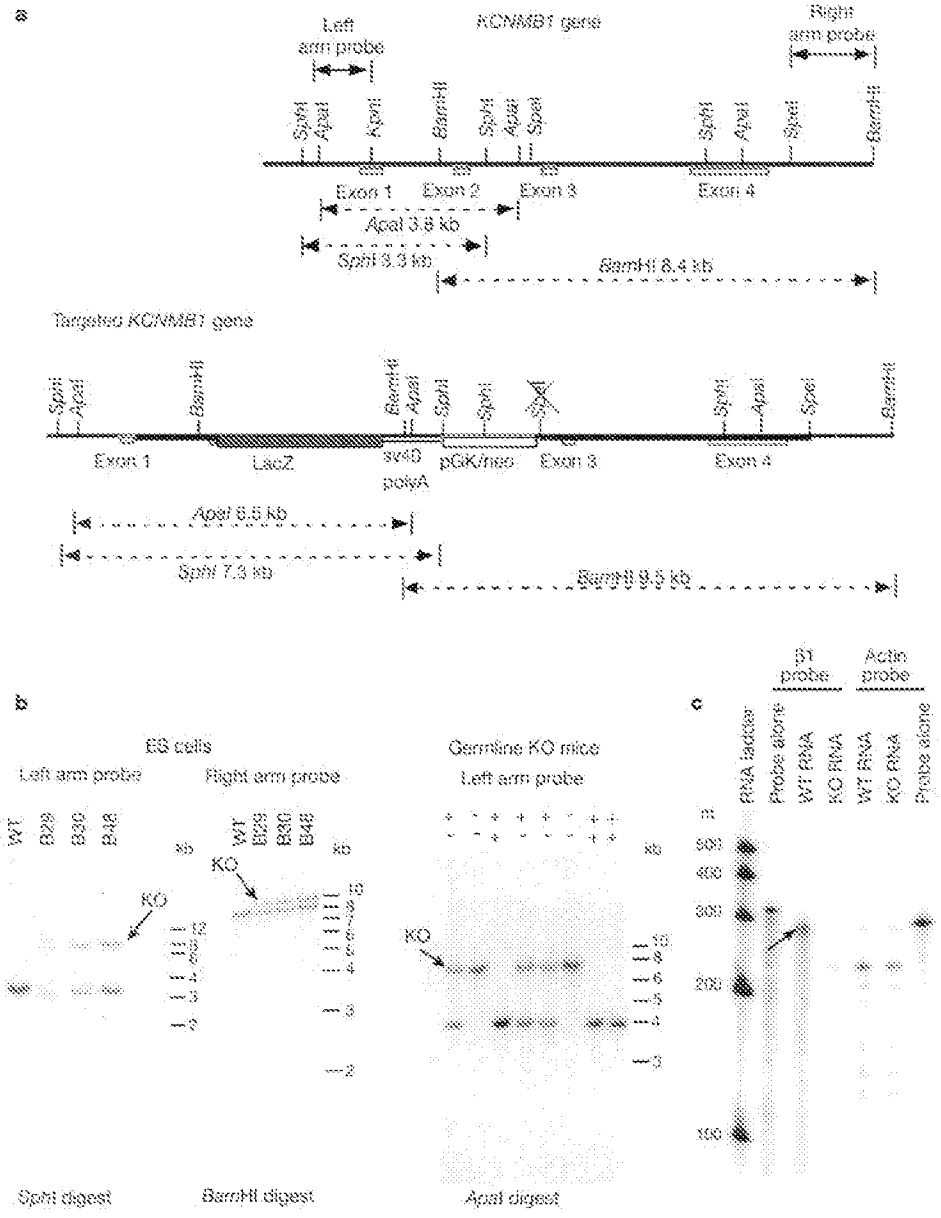

Figure 1 Generation of β1 gene knockout mice. **a**, Restriction map of the β1 gene locus (top) and the targeted β1 gene (bottom). Thick line in the targeted locus map designates the regions used for the targeting vector. **b**, Southern and RNA analysis of the gene knockouts. Left, Southern analysis of ES cells using the probes designated in **a**. Lanes contain genomic DNA from control ES cells (WT) or the β1-targeted ES clones (B29, B30 and B48). Middle, genomic Southern analysis of β1 knockout siblings derived from clone B30 heterozygous parent matings. Lanes are labelled with the genotypes. **c**, RNase protection assay of β1 expression from control (WT) and β1 knockout (KO) RNA. The antisense β1 probe hybridizes to a specific 290-nucleotide (nt) product in WT RNA (arrow) but not to the β1 knockout RNA. A mouse β-actin RNA probe hybridizes to a specific 245-nt product in both WT and KO RNA samples.

was enriched in aortic smooth muscle but absent from brain[19]. Expression of β1 was also observed in other tissues, including smooth muscle of the bladder, trachea, bronchi and the digestive tract (trachea, stomach and colon staining are shown in Fig. 2d–f, respectively).

β1 knockout BK channels have reduced Ca^{2+} sensitivity

The fact that the β1 subunit is restricted in expression to smooth muscle cells suggested a unique role of the β1 subunit in Ca^{2+} signalling in smooth muscle. To confirm that the arterial smooth muscle from the knockout mice lacked a functional β1 subunit, the sensitivity of BK channels to calcium and the BK/β1 channel agonist DHS-1 were examined in inside-out patches from freshly isolated cerebral artery myocytes at physiological membrane potentials (−40 mV) for pressurized arteries[4,5]. Figure 3 compares single BK channel recordings of excised patches from knockout and control arterial smooth muscle. BK channels were exposed to cytoplasmic calcium concentrations of 3 and 10 μM, concentrations within the range predicted for calcium-spark-evoked activation of BK channels in arterial smooth muscle[20]. At −40 mV, control BK channels have a significant open probability when activated by 3 μM ($Po = 0.47 \pm 0.2$) and by 10 μM Ca^{2+} ($Po = 0.60 \pm 0.2$). In contrast, BK channels from KO cells had an open probability at least 100-fold lower at −40 mV ($Po = 0.003 \pm 0.002, 0.007 \pm 0.002$ for 3 and 10 μM calcium, respectively). Increasing the membrane voltage to +40 mV increases the open probability in both control and KO channels, but the control BK channels still have significantly higher open probability than the BK channels in the knockout cells (control, $Po = 0.89 \pm 0.03, 0.78 \pm 0.073$; knockout, $Po = 0.22 \pm 0.07, 0.53 \pm 0.06$, for 3 and 10 μM calcium, respectively).

The agonist DHS-1 significantly increases the open probability of the channel when associated with a β1 subunit[20] and provides a pharmacological probe for the presence of BK α/β1-assembled subunits. Figure 4a shows that application of DHS-1 to the bath causes a significant increase in open probability in control mice ($Po_{control} = 0.11 \pm 0.1$ versus $Po_{control+DHS1} = 0.25 \pm 0.1$; $P < 0.05$, $n = 3$, −40 mV). DHS-1, however, had no effect on the BK channels from β1 knockout mice ($Po_{KO} = 0.005 \pm 0.001$ versus $Po_{KO+DHS1} = 0.007 \pm 0.001$; $P > 0.4$, $n = 3$, −40 mV). Neither BK channel density, as assayed from the average number of channels detected in patches (Fig. 4b), nor the BK channel unitary conductance (γ), was affected in the knockout ($\gamma_{KO} = 209 \pm 14$ pS, $\gamma_{control} = 215 \pm 1.1$ pS, $n = 3$ patches each, from −90 to +60 mV in symmetric 140 mM K, $P > 0.4$). The decreased open probability and insensitivity to DHS-1 support the conclusion that BK channels in normal cerebrovascular smooth muscle consist primarily of BK α/β1 subunits, whereas the β1 knockout mice contain BK channels lacking an associated β1 subunit. This is consistent with the LacZ staining demonstrating β1 subunit expression in cerebral artery smooth muscle (Fig. 2).

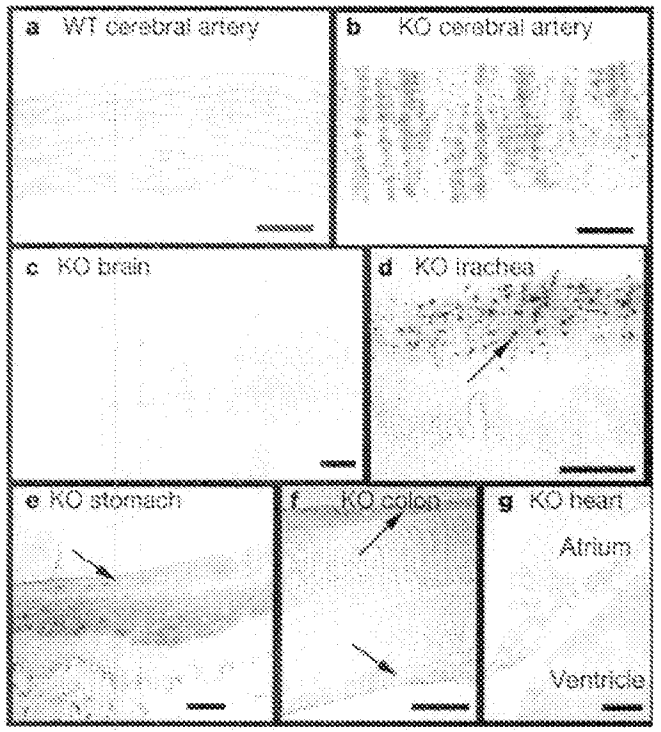

Figure 2 Detection of lacZ gene expression from β1 gene-targeted mice. Staining of whole-mount tissue (**a**, **b**) and frozen sections (**c**–**f**) from knockout (KO) and control (WT) mice. Blue staining (arrows) labels regions of β1 expression. Scale bars: **a**, **b**, **d**, **e**, 50 μm; **c**, 1 mm; **f**, 200 μm; **g**, 500 μm. Tissues are counterstained with Orange-G in **d**–**g** and with Eosin Y in **c**.

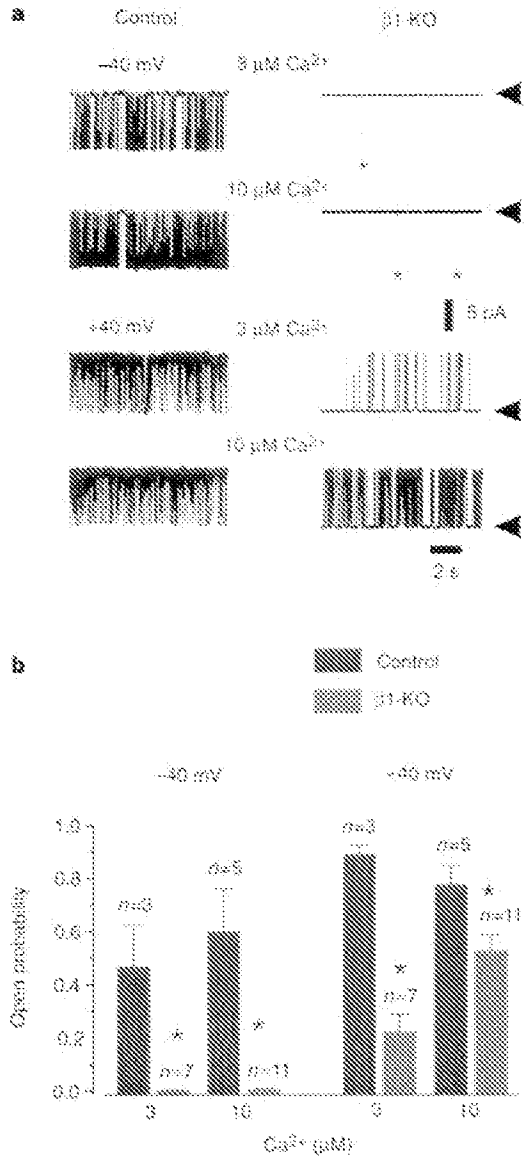

Figure 3 Ca^{2+} and voltage dependence of BK channels in cerebral artery myocytes from control and β1-KO animals. **a**, Single channel recordings in inside-out patches held at −40 and +40 mV, and with 3 and 10 μM free Ca^{2+}. Arrows, closed state. Asterisks, brief channel openings in β1-KO patches. **b**, BK channel open probability in control and β1-KO animals at two different voltages (−40 and +40 mV) and two Ca^{2+} concentrations (3 and 10 μM). Asterisks denote statistically significant difference from controls.

β1 subunit couples calcium sparks to BK channel activation

The open probability of BK channels is low at physiological membrane potentials (−40 mV) and average arterial smooth muscle calcium of pressurized cerebral arteries[3]. A calcium spark can elevate the *Po* of nearby BK channels 10^4 to 10^6-fold to produce a significant transient membrane potential hyperpolarization (20 mV)[2,26]. To explore the role of the β1 subunit in the communication of calcium sparks to BK channels, we measured calcium sparks and whole-cell potassium currents simultaneously in isolated cerebral artery myocytes[20]. Figure 5a illustrates the life cycle of a spark from a β1 knockout mouse, peaking in around 20 ms and then decaying over 200 ms, very similar to control myocytes[20]. Figure 5b shows a representative simultaneous recording of whole-cell current (in blue) and sparks (in red and green). Transient BK current amplitude increases with Ca^{2+} spark amplitude in both control and knockout cells (Fig. 5c). However, the mean transient BK current amplitude was around one-sixth of control amplitude for a given Ca^{2+} spark amplitude. Furthermore, there was a striking difference in the ability of Ca^{2+} sparks to activate transient BK currents. In the control, essentially every Ca^{2+} spark evoked a transient BK current at −40 mV. In contrast, in the knockout 35% of the sparks failed to evoke a detectable BK current (Fig. 5d). BK channel density, Ca^{2+} spark amplitude (control = 1.65 ± 0.03 (n = 94 from 6 cells), knockout = 1.77 ± 0.05 (n = 71 from 7 cells)), and spark frequency (control = 1.41 ± 0.2 Hz (6 cells), and knockout = 1.35 ± 0.3 (7 cells)) were unaltered in the knockout. These results indicate that the overall BK channel activity during Ca^{2+} sparks is reduced at least 12-fold in the knockout, consistent with the diminished Ca^{2+} sensitivity of BK channels (Figs 3 and 4).

Lack of β1 elevates arterial tone and blood pressure

BK channels lacking the β1 subunit are present and functional in arteries from knockout mice, albeit less sensitive to activating calcium. To understand the physiological consequences of the reduced coupling between calcium sparks and BK channel activation in the β1 knockouts, we evaluated the effect of pressure on arterial diameter. Elevation of intravascular pressure constricts small arteries, including cerebral arteries[4,20,21]. Cerebral arteries that lack the β1 subunit are significantly more constricted at a given pressure than are control arteries (Fig. 6a–c). These results indicate that the lack of the β1 subunit leads to an elevation in arterial tone.

The contribution of the β1 subunit to the regulation of arterial tone can be evaluated by examining the effects of the BK inhibitor iberiotoxin (IBTX) on arterial diameter. IBTX caused a 74% increase in arterial tone in the control (Fig. 6d, f). In contrast, IBTX did not affect knockout cerebral arteries (Fig. 6e, f). These results indicate that BK channels lacking the β1 subunit are unable to contribute to the regulation of arterial tone.

Unless systemic physiological control mechanisms can compensate for the increased arterial tone, arterial blood pressure should be elevated in mice lacking the BK β1 subunit. The mean arterial blood pressure of the knockout mice was indeed elevated and comparable to transgenic mice with compromised endothelial function[20]. The knockout lines were bred from a mixture of 129svj mice, used to derive the embryonic stem cells, and C57BL mice, used as the initial breeder mates. We chose to compare the mean arterial pressure of our knockout mice to the mean arterial pressure of 129svj control mice to generate the most stringent test for evidence of elevated blood pressure, as the 129svj mice have a higher mean arterial blood pressure than any other strain[23]. The knockout mice exhibited an increase in mean blood pressure, even over the pure 129svj mice (Fig. 7a). These data indicate that the increased arterial tone leads to an elevation in blood pressure.

In humans, long-standing essential hypertension induces significant left ventricular hypertrophy and leads to heart enlargement[24].

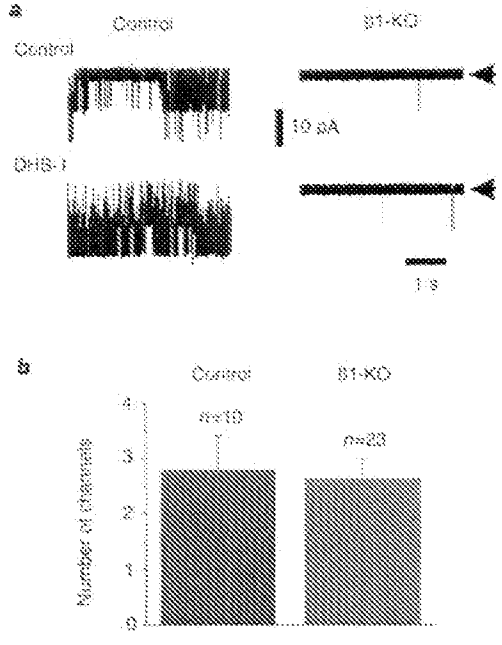

Figure 4 DHS-1 sensitivity and density of BK channels in cerebral arteries myocytes from control and β1-KO animals. **a**, Single channel recordings at −40 mV and 10 μM Ca^{2+}, before and after the addition of 100 nM DHS-1. **b**, Average number of channels per patch excised from control and β1-KO myocytes (10 and 23 patches, respectively). No significant difference was found between control and β1-KO patches. Pipette resistances were 8–11 MΩ.

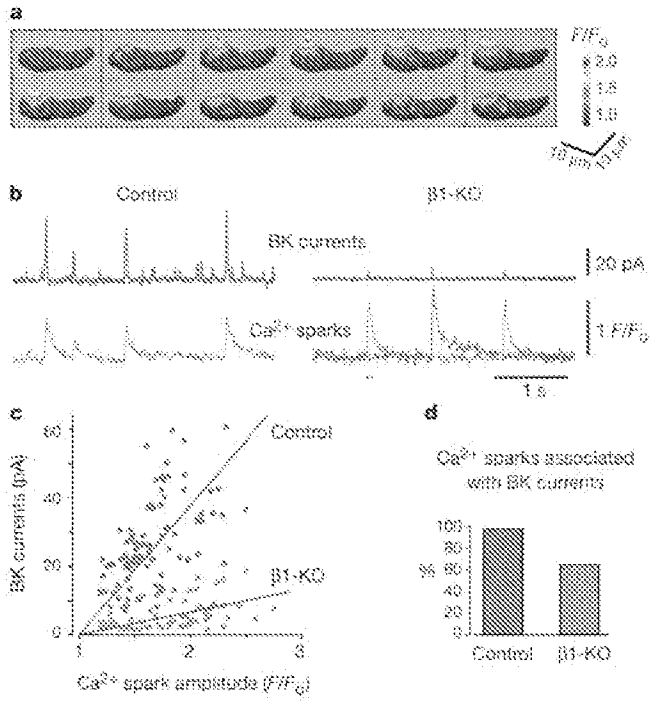

Figure 5 Decreased coupling of calcium sparks to BK channels in β1-KO myocytes. **a**, Consecutive pseudocolor three-dimensional images of a β1-KO cell obtained every 8.33 ms. **b**, Simultaneous BK current (blue) and Ca^{2+} spark measurements (fractional fluorescence, F/F_0) from a control cell with one spark site and a β1-KO cell with two spark sites (red and green) (−40 mV). Red bar indicates the segment of the red trace illustrated in **a**. **c**, Relationships between BK current and Ca^{2+} spark amplitudes in control cells (blue, 94 sparks, 6 cells) and β1-KO cells (red, 71 sparks, 7 cells). Lines represent linear regression fit ($slope_{control}$ = 37.8 ± 1.6 versus slope β1-KO = 6.7 ± 0.5, $P < 0.001$). **d**, Percentage of Ca^{2+} sparks causing transient BK currents in control (blue) and β1-KO (red) cells.

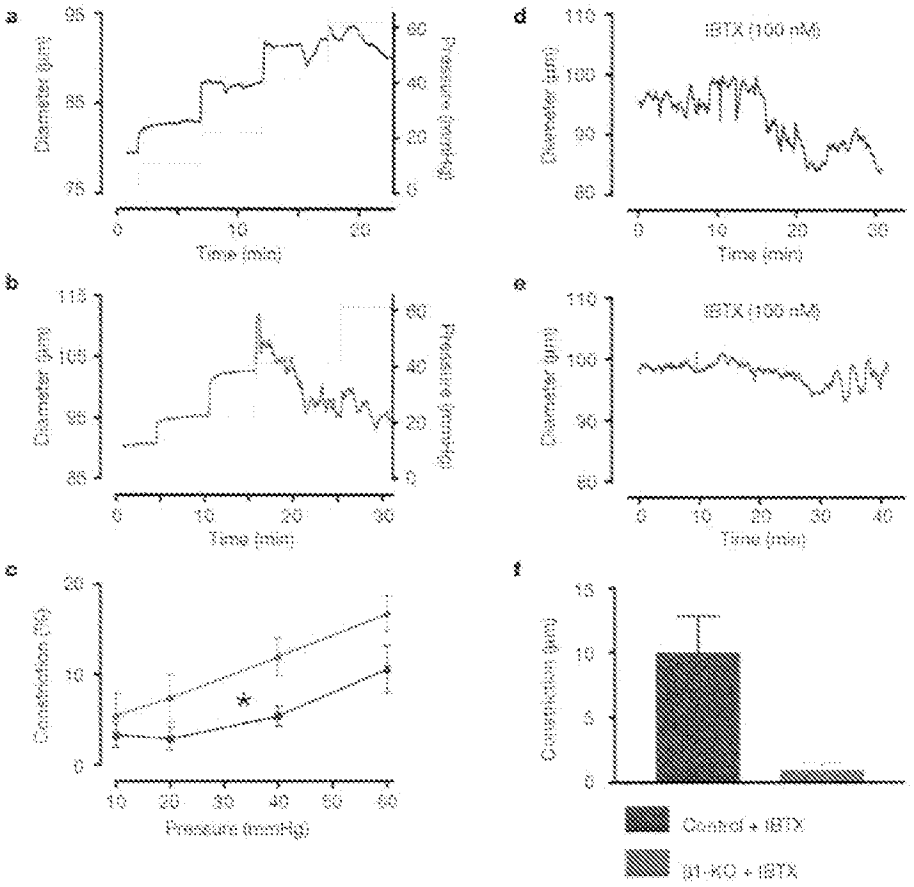

Figure 6 β1-KO cerebral arteries are more constricted to pressure. **a**, Control, blue; **b**, β1-KO, red; **c**, summary, $P < 0.05$). They are also insensitive to the BK channel blocker iberiotoxin (**d**, control, blue; **e**, β1-KO, red; **f**, summary). Grey lines indicate pressure levels in **a** and **b**.

To measure heart enlargement, we normalized the heart weight to animal body weight. Knockout mice had larger heart-to-body weight ratios than 129sv] mice (Fig. 7b). Furthermore, at the electron microscopy level, there were no obvious differences in ultrastructure of the cardiomyocytes between the 129sv] control hearts and the β1 knockout hearts (Fig. 7c). Myofibrillar organization, mitochondrial prevalence and structure, and glycogen content appeared similar in control and knockout mice. There was no evidence of necrosis or fibrosis. As heart muscle ultrastructure appeared normal, these findings are consistent with heart enlargement caused by uncomplicated essential hypertension.

Discussion

The BK channel is the only member of the voltage-dependent potassium channel family that is activated by both voltage and calcium. This makes it particularly suited to integrate calcium and voltage signals to modulate membrane excitability in a variety of cell types. But the tissues that express BK channels have diverse functions with considerable differences in excitability and Ca^{2+} signalling. Our results support the concept that the β1 subunit is required specifically to tune BK channel properties to the needs of an arterial smooth muscle cell. The increased sensitivity to calcium conferred by the β1 subunit is required for the BK channel to translate calcium sparks to membrane potential hyperpolarization. The decreased coupling of Ca^{2+} to channel activity extrapolated well to the functional defects observed in the intact artery and whole animal. Moreover, end organ pathology observed in chronic hypertension, such as myocardial hypertrophy, was also observed in the BK β1 knockout mice. The BK β1 knockout mouse therefore presents a unique model, wherein a clearly defined molecular defect could be used to study the secondary effects of hypertension. Moreover, the β1 subunit gene should provide a candidate genetic locus for human hypertension.

Not only was BK channel activity reduced when the β1 subunit was absent, as detected by the reduced size of transient BK currents, but many calcium sparks failed to cause detectable currents. The most direct explanation is that the calcium sensitivity of the BK channel lacking β1 is reduced sufficiently so that the channel open probability during a spark is too low to cause a detectable current (one single BK channel amplitude at −40 mV, or 2 pA). The effect is to uncouple BK channel activation from calcium spark signals. Further evidence of uncoupling was the lack of effect of iberiotoxin on arterial tone in β1 knockout arteries. It has been estimated that BK channels in the presence of β1 subunits appose close enough to calcium release sites to detect 10–100 μM effective calcium concentrations[28]. In 10 μM calcium, cloned vascular smooth muscle BK channels lacking a β1 subunit have a voltage for half activation of around +30 mV[29], and have a very low open probability at −40 mV, the membrane potential of smooth muscle in intact pressurized arteries. In contrast, the same channels containing the β1 subunit have activation voltages 70 mV more negative[29], at approximately the resting membrane potential for smooth muscle cells (−40 mV). Thus, the reduced apparent calcium sensitivity can explain the difference in spark/BK current coupling between the knockout mice and their controls. However, this does not exclude the possibility that the β1 subunit may have other effects on BK channel properties. For example, β1 subunits could be required for subcellular localization of BK channels to calcium release sites or modifying BK channel phosphorylation-dependent regulation.

The BK channel regulates arterial diameter and mediates the response to a number of smooth muscle relaxants including nitric

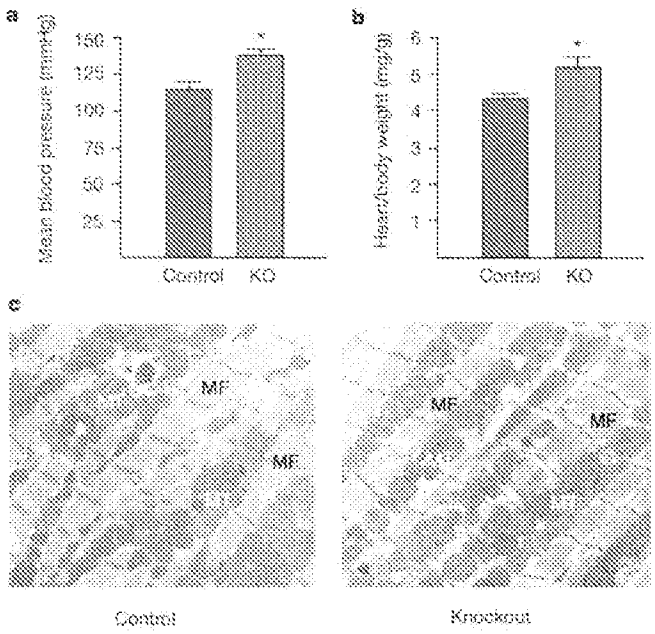

Figure 7 β1-KO mice show symptoms of hypertension. **a**, Mean blood pressure of β1-KO and control 129svj mice. Control average blood pressure is 114 ± 6.0 mm Hg ($n = 6$). KO average blood pressure is 134 ± 5.1 mm Hg ($n = 6$), $P = 0.023$. **b**, Heart/body weight measurements comparing β1-KO and control mice. KO is 5.0 ± 0.22 mg/g ($n = 11$); controls are 4.35 ± 0.15 mg/g ($n = 8$), $P = 0.037$. **c**, Electron microscopy of heart tissue from β1-KO and control mice. Examples of myofibril (MF) and mitochondria (M) are labelled.

oxide[36–38]. Our results support the concept that the calcium sensitivity of the BK channel is fine tuned to respond to calcium signals unique to the physiology of a given cell type. In the case of arterial smooth muscle, the β1 subunit is essential for the effective coupling of calcium sparks to BK channels, thereby enabling BK channel regulation of arterial smooth muscle tone. In different tissues, other β-subunit family members may serve similar roles. Furthermore, we propose that other agents, such cyclic AMP- and cGMP-mediated vasodilators, which modulate the calcium sensitivity of the BK channels, can dynamically match calcium signals to BK channel activation to regulate cell function. □

Methods

β1 knockout mice

To generate a left arm fragment, we amplified a 129svj/lambda FixII genomic clone of the β1 gene with a *5′ primer* that overlapped the β1 seventh codon (methionine) and created a flanking *EpoI* site. The *5′ primer* introduced a *NotI* site that annealed 1.6 kilobases (kb) upstream (in the first exon[39]). The left arm PCR product was ligated in a three-way ligation with a *EpoI/SalI* fragment containing the lacZ/SV40 polyA sequence and a *MluI/XhoI* cut pPNT knockout vector[40]. The right arm was a 5.4-kb *SpeI* genomic fragment (Fig. 1) cloned into the *XhoI* site of the pPNT knockout vector[40]. The construct was electroporated into 129svj E8 cells. We screened candidate clones by genomic Southern analysis of the short arm (Fig. 1) and confirmed them by Southern analysis of the long arm. Targeted E8 cells were used to generate germline carrying mice[41]. Germline siblings (mixed 129svj/C57BL background) were mated to produce homozygous stocks. 129svj mice were used as controls for blood pressure and heart/body weight measurements and either 129svj or F_1 progeny from a 129svj/C57BL cross were used for other experiments. Animals used were males between 3 and 8 months of age for blood pressure and heart size measurements, or either males or females of 3–8-month age range for cell physiology experiments.

DNA and RNA analysis

Genomic Southern analysis was as described[42]. To make an antisense β1 RNA, nucleotides 1–290 of the coding portion of the mouse β1 complementary DNA were subcloned into the vector pGX[43] and transcribed using the T7 RNA polymerase promoter. RNAse protection assays were conducted using the Ambion RPAII kit according to the manufacturer's protocols (Ambion). Hybridization was conducted with 2 mg of poly(A) purified stomach RNA to detect β1 or 200 ng total stomach RNA to detect actin.

LacZ staining of tissue and electron microscopy

Frozen sections (16 μm) were fixed in 0.2% glutaraldehyde in PBS, washed three times in PBS and then stained for β-galactosidase activity as described[44]. The tissues were counterstained with Eosin Y or Orange G. Electron microscopy of heart tissue was done as described[39].

Tissue preparation

Knockout or control mice (129/sv or 129/svXB6) of either sex were killed by exsanguination while under deep pentobarbital anaesthesia (intraperitoneal; 150 mg kg^{-1} body weight). Cerebral arteries were rapidly dissected while the brain was submerged in cold (4 °C) oxygenated (95% O_2/5% CO_2) physiological saline solution (PSS) of the following composition (in mmol l^{-1}): 118.5 NaCl, 4.7 KCl, 24 $NaHCO_3$, 1.18 KH_2PO_4, 2.5 $CaCl_2$, 1.2 $MgCl_2$, 0.023 EDTA, 11 glucose.

Diameter measurements

We measured the diameter of middle cerebral artery segments by video edge detection, in oxygenated PSS at 37 °C and pH 7.4 (refs 1, 2, 4, 5, 44, 45). The diameters (at 10 mm Hg) from control and β1-knockout arteries were 92.0 ± 2.5 μm ($n = 11$) and 88.5 ± 2.7 μm, $n = 12$) respectively. Pressure-induced constrictions are expressed as a per cent decrease of the diameters in Ca^{2+}-free PSS containing 1 μmol l^{-1} nisoldipine.

Electrical and optical measurements

Single smooth muscle cells were isolated from cerebral arteries by digestion in 0.5 mg ml^{-1} papain and 1 mg ml^{-1} dithioerythritol for 10 min, and then transferred for a second digestion in 1 mg ml^{-1} collagenase (type F and type H in a 70%–30% mixture) for 7 min[5,6,46]. Membrane current and calcium sparks were measured simultaneously in myocytes loaded with the Ca^{2+} indicator fluo-4, using perforated patch, whole-cell configuration and a laser (two-dimensional) scanning confocal microscope[5,46]. The bathing solution was (in mM): 134 NaCl, 6 KCl, 1 $MgCl_2$, 2 $CaCl_2$, 10 glucose, 10 HEPES pH 7.4, with 5 μM wortmannin to minimize contraction. The pipette solution contained (in mM): 110 K-aspartate, 30 KCl, 10 NaCl, 1 $MgCl_2$, 10 HEPES (pH 7.2) and 250 mg ml^{-1} amphotericin B. Single BK channel currents were recorded in inside-out patches, exposed to calibrated Ca^{2+} buffered solutions (in mM): 140 KCl, 10 HEPES (pH 7.2), 1 Mg^{2+}, 5 HEDTA, and 5 or 10 μM Ca^{2+} free (adjusted with Ca^{2+} electrodes). Pipette solution contained (in mM): 140 KCl, 10 HEPES (pH 7.2), 1 Mg^{2+}, 5 HEDTA and 10 μM Ca^{2+}. Single-channel currents were recorded over 3–10 min at steady potentials of −40 and +40 mV at room temperature. Currents were filtered at 1 kHz and digitized at 4 kHz.

Blood pressure analysis and heart/body weight ratios

Mean arterial blood pressures were obtained using arterial catheters surgically inserted into the left carotid artery as described[47]. Following surgery, animals were allowed to recover for 24 h, and mean arterial blood pressure was measured every 8 s for 30 min. The average mean arterial blood pressure values obtained during the sampling period was calculated for each animal. All measurements were performed with the animal resting quietly. To obtain heart weights, isolated beating hearts were dissected free and allowed to expel the blood volume in saline solution. Hearts were transferred to 1 M KCl solution for 15–30 s, washed in saline, blotted free of excess solution and weighed. For blood pressure measurements the knockout animals had a mean age of 17.7 ± 2.2 weeks, and control animals were 20.2 ± 3.5 weeks. For heart/body weights, the animals were matched well for body weights (knockout, 32.1 ± 1.8 versus controls, 31.7 ± 1.2 g) although not ideally for age (knockout 15.6 ± 0.9 weeks). Our data and others show no correlation between increasing age and heart/body weights in normal animals[48,49].

Data analysis

Image and BK current analysis was performed using a custom written analysis program using Interactive Data Language software (Research Systems Inc). Baseline fluorescence (F_0) was determined by averaging 30 images with no activity. Fractional fluorescence (F/F_0) versus time traces were obtained by averaging F/F_0 from a box region of 2.2 × 2.2 μm centred in the active area of interest (Ca^{2+} sparks sites). Transient BK currents caused by Ca^{2+} sparks with amplitudes bigger than a single BK channel opening (2 pA, at −40 mV) were considered for further analysis. Number of channels (N) × open probability (Po) of single channel currents was calculated from all points histograms and divided by the number of channels present in the patch to obtain Po values.

Statistical analysis

Results are expressed as means ± s.e.m., where applicable. Comparisons between control and knockout data were done with the unpaired two-tailed student's *t*-test.

Received 6 July; accepted 30 August 2000.

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Acknowledgements

We thank B. K. Kobilka of Stanford University for advice; Y. Chen in the Stanford Transgenic Mouse facility for the blastocyst injections; G. Mawe for advice and assistance on isolated artery images; S. Brett Welsh for genotyping and technical assistance; D. Hill-Eubanks for comments on the manuscript and D. P. Regula for paraffin sections of hearts. DHS-1 was provided by Merck Research Laboratories. This work was supported by a grant from the Federation for Anesthesia Education and Research to A.J.P. and by grants from the National Institutes of Health (H.L.B.J. and D.D.K.), National Science Foundation, Totman Medical Trust for Cerebrovascular Research to M.T.N., and an American Heart Association Fellowship to G.P. R.W.A. is an investigator with the Howard Hughes Medical Institute.

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